ISOLATION AND CHARACTERIZATION OF THE AGROBACTERIUM TUMEFACIENS FROM ALMOND NURSERIES IN CHLEF REGION IN WESTERN ALGERIA

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Abstract

Crown gall is one of the destructive diseases and occurs worldwide. It is considered to be a disease of great economic importance in almond and other stone fruit tree nurseries due to the extensive losses. Based on their morphological characteristics on MacConkey medium and YMA medium, 10 isolates were selected on colonies of these isolates after 48 h at 28°C were circular, convex with smooth, translucent and easily suspended in water. The bacterial cells were rod shaped with rounded ends and were either single or in pairs. The isolates were Gram negative, the optimum growth was between 25 and 27°C.
All strains are positive for mobility, catalase, oxydase. On the other hand, these isolates had all oxidized the lactose to 3-ketolactose. On the other hand, all *Agrobacterium* strains oxidized Sucrose, D-mannitol, D-sorbitol, Indol, inositol, Melibioze, D-galactose, L arabinose, Rhamnose, Amygdalin, Lactose and Glucose. Furthermore, the isolates transform also the arginin, lysin, ornithin, gelatin and starch. The pathogenic nature of the organism was confirmed by a bioassay on carrot disks. Additionally, Koch’s postulates for all isolates were also fulfilled.

Keywords: Agrobacterium tumefaciens, Almond, pathogenecity tests, biochemical characterization

1. Introduction:

Crown gall caused by *Agrobacterium tumefaciens* (Smith and Townsend) Conn., is widespread and has worldwide distribution (Matthow et al., 2003). It is considered to be a disease of great economic importance in almond and other stone fruit tree nurseries due to the extensive losses

(Kersters, 1984; Woese, 1987; Bouzar et al., 1991; Thakur et al., 2007). The galled seedlings are generally refused for sell. The causal agent *A. tumefaciens* is ubiquitous in soil. It infects the roots of dicotyledonous plants through lesions or injuries and hence systematically infects the whole plant. These wounds may be caused by biological agent such as nematodes, insects or mechanical tools (Kersters, 1984; Woese, 1987; Bouzar et al., 1991).

Crown gall infection process involves the transfer of a specific region of TDNA (Tumor Inducing) Ti plasmid to the plant cell which is then stably integrated in host genome. (Matthow et al., 2003; Cazelles et al., 2008). As a result, fundamental physiological changes occur in the infected plant tissues such as over expression of the biosynthesis of phytohormones auxins and cytokinins and other unusual chemicals of low molecular weight called opines, which play a key role in the symptomatology and epidemiology of the disease (Zambryski, 1989).

The major synthesized opines (octapines and nopalines) are released in the intercellular spaces of the infected plant tissues. These compounds are utilized primarily as carbon and nitrogen sources for the bacteria. Furthermore, opines participate in the conjugal transfert of Ti plasmid from the pathogenic strains to avirulent recipient (Goldman et al., 1969; Desseaux et al., 1992). The crown galled plants are also less vigorous with chlorosis and are subjected to other secondary attacks such as fungal infection.

This study was undertaken to isolate and characterize the *Agrobacterium tumefaciens* isolates from both the rhizosphere and seedlings almond plant from stone fruit tree nurseries using the pathogenecity test, growth characteristics on semi selective media, biochemical and physiological patterns (Madigan et Martinko, 2007).

2. Materials and Methods:

2.1. Samples collection and isolation of *Agrobacterium tumeficiens* from infected seedlings:

The survey was conducted between October and November 2010 in stone fruit tree nursery located in Chlef region in western Algeria. The samples from almond seedlings plants presenting tumors were collected in a polyethylene bags. The fresh galls were detached carefully and washed in running water to remove soil. Small sections of living tissues were chopped with a sterilized scalpel and immersed in sterilized distilled water and were left standing for overnight at room temperature. A loopful of the suspension was streaked on two media namely YMA, and MacConkey agar, all inoculated plates were incubated for 1 week at 28°C. The colonies that are representative of *Agrobacterium* were restreaked on MacConkey medium for purification. The purified colonies were then stored at 4°C on YMA medium supplemented with 0.5% $CaCO_3$.

2.2. Characterization of *A. tumeficiens:*

2.2.1. Cultural and morphological characteristics:

Among the cultural and morphologic characters maintained for the selection of *A. tumefaciens* strains include shape, size, color, colony surface, elevation, margin type, consistency, translucency or opaqueness.

2.2.2. Biochemical and physiological tests:

The biochemical and physiological of isolates was conducted according to Moore et al (1988). For each strain of *Agrobacterium* the following tests including Gram staining motility, catalase, oxidase, urease, citrate utilization production, arginine dihydrolase activity, nitrate reduction, aerobic growth, gelatin and hydrogen sulfide production. Bacterial strains were tested for their ability to oxidize carbon substrates including Sucrose, D-mannitol, D-sorbitol Indol, inositol, Melibioze, L arabinose, Rhamnose, Glucose, starch hydrolysis. Furthermore, bacterial strains were tested for their ability for oxidation of lactose to 3-ketolase using the benedict's test (Bernaerts and Ley, 1963). All the isolates with a positive reaction are considered as *A. tumefaciens* biovar 1(Bouzar, 1993). Growth at 37°C and the tolerance to 2% NaCl were also tested. The growth was evaluated after 48h at 28°C.

Discs prepared from carrot (*Daucus carrota* L.) were sterilized and placed in petri dish with moist filter paper. Each disc was pulverized with 100µl of dense inoculum (10^9cfu/ml) (Aysan et al., 2003; Soriful et al., 2010). The plates were incubated at room temperature under dark conditions. After 3 weeks, the discs were checked for microtumors.

2.2.3. Antibiotics resistance tests:

The antibiotics resistance test was performed on the Mueller-Hinton agar using the in vitro disc diffusion method. The *A. tumefaciens* strains were subjected to the following antibiotics: Vancomycine3µg, Kanamycine 30 µg, Gentamycin 10 µg. The inhibition zone was measured after 48h of incubation at 28°C.

3. Results:

The present study was conducted to determine the biochemical, physiological and pathogenecity of *A. tumefaciens* strains randomly isolated from tumors on stone fruits seedlings in nurseries in chlef region in western Algeria. Based on their morphological characteristics on MacConkey and YMA, 20 isolates were selected on colonies of these isolates after 48 h at 28°C were circular, convex with smooth, translucent, 1-1.5mm in diameter and easily suspended in water.

The bacterial cells were rod shaped with rounded ends and were either single or in pairs. Their average size was 0.7x2.3µm; they were mobile due to peritrichous flagella. The isolates were Gram negative, the optimum

growth was between 25 and 27°C. The Biochemical features of the *A. tumefaciens* strains are presented in table 1. All strains are positive for mobility, catalase and oxydase. These isolates had also all oxidized the lactose to 3-ketolactose. Moreover, all *Agrobacterium* strains oxidized Sucrose, D-mannitol, D-sorbitol, Indol, inositol, Melibioze, L arabinose, Rhamnose, Glucose, lactose and starch hydrolysis. Furthermore, the isolates transform also the arginin, lysin, ornithin, gelatin and starch (Table1).

Table1. Phenotypic characteristics of selected strains of *A. tumefaciens* collected from almond seedlings.

Biochemical tests	Strains									
	BSt 01	BSt 02	BSt 03	BSt 04	BSt 05	BSt 06	BSt 07	BSt 08	BSt 09	BSt 010
Gram stain	-	-	-	-	-	-	-	-	-	-
Mobility test	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Oxydase	+	+	+	+	+	+	+	+	+	+
Growth in 2% NaCl	+	+	+	+	+	+	+	+	+	+
Growth on 35°C	+	+	+	-	+	-	+	+	+	-
Production of H_2S	-	-	-	-	-	-	-	-	-	-
3-Ketolacotose production	+	+	+	+	+	+	+	+	+	+
Nitrate production	+	+	+	+	+	+	+	+	+	+
Carbohydrate utilization:										
Sucrose	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	+	+	+	+
D-sorbitol	+	+	+	+	+	+	+	+	+	+
Indol	+	+	+	+	+	+	+	+	+	+
inositol	+	+	+	+	+	+	+	+	+	+
Melibioze	+	+	+	+	+	+	+	+	+	+
D-galactose	+	+	+	+	+	+	+	+	+	+
L arabinose	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+
Amygdalin	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Arginin utilization	+	+	+	+	+	+	+	+	+	+
Lysine utilization	+	+	+	+	+	+	+	+	+	+
Citrate utilization	-	-	-	-	-	-	-	-	-	-
Ornithin utilization	+	+	+	+	+	+	+	+	+	+
gelatin liquefaction	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+
Urea test	+	+	+	+	+	+	+	+	+	+
Antibiotics resistance test:										
Vancomycine	R	R	R	R	R	R	R	R	R	R
Kanamycine	S	S	S	S	S	S	S	S	S	S
Gentamycine	R	R	R	R	R	R	R	R	R	R
Growth on pH=5.5.	++	+	++	+	+	+	++	+++	++	+
Growth on PH=7	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Antibiogram test showed that all *A. tumefaciens* presented sensitivity towards kanamycin and were resistant towards Vancomycine and Gentamycine. Concerning the pathogenecity test, all strains had induced

tumor formation when inoculated in carrot discs. Smalls galls were observed on carrot and potato discs after 4 weeks of inoculation. No symptoms were noted on un-inoculated controls indicating that these strains isolated form almond seedlings were all pathogenic.

4. **Discussion**:

The purpose of this study is to isolate and characterize the *A.tumefaciens* strains collected from almond seedling presenting crow gall from stone fruit nursery in chlef region in western Algeria. Bouzar et al (1991) in previous studies conducted in Algeria have found that *A. tumefaciens* pose a serious threat for stone fruit nurseries. On the basis of the results obtained with conventional analysis, the 10 isolates selected from diseased seedlings were identified as *Agrobacterium tumefaciens.*

The results of some phenotypic characters, such as the morphology of cells and colonies, growth on 35°C, tolerance to NaCl, and production of acids from many carbon sources including Sucrose, D-mannitol, D-sorbitol, Indol, inositol, Melibioze, L arabinose, Rhamnose, Glucose, lactose and starch hydrolysis was in accordance with typical pattern of A. tumefaciens. Moreover, the confirmation of *A. tumefaciens* was conducted based on specific tests including growth on MacConkey medium and production of 3-Ketolactose tests. Such tests have already been performed and their obtained results are in agreement with our results (Chen et al., 1999; Bouzar et al., 1993). The antibiotic test revealed that all isolates of *A. tumefaciens* were resistant to Vancomycine and Gentamycine. Sensitivity however was noted towards Kanamycin. In previous study, Hafiz (1986) obtained an effective protection against *A.tumefaciens* when using tetracycline. Marja et al (2004) confirmed also the sensitivity of *A. tumefaciens* strains to many of antibiotics including tetracycline and kanamycine. On the other hand, the tumorigenic capacity obtained with in vitro test confirmed the affiliation of these strains to *A. tumefaciens* Biovar 1 as classified by Moore et al (2001). In fact, the differences in response between the isolates could reflect natural variability.

This bacterial disease represents a threat for the production of stone fruit seedlings in chlef region. Hence, it would be desirable to monitor soils and other hosts other than almond, in order to ascertain the diffusion of this disease and verify epidemiological aspects and establish a control strategy. Crown gall as most stone fruit disease is difficult to control using bactericidal compounds. Hence, the rules to be followed are similar to those used as strategies for control other plant bacterial disease. The first rule is to follow the prevention and sanitation measures. The best way to ovoid its introduction is by using healthy materials. The grafting procedure should be performed under sanitary conditions. The soil treatment might be also an effective measure for limiting the expansion of the disease.

Moreover, the *Agrobacterium* is systematically disseminated in several plant species (Burr, 1978) which facilitate their propagation, hence the early detection could be essential to ovoid disease spread by using polymerase chain for the amplification of certain conserved regions of Ti plasmids (Sawada et al., 1993; Haas et al., 1995). This method could be used for the production of certified pathogen free propagation seedlings. On the other hand, the use of biocontrol agents particularly among the avirulent *Agrobacterium* strains (Farrand, 1995; Moore and Warren, 1979) and the identification of other bacterial antagonist species could be an additional tool as an effective means for the control of the crown gall.

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